Cell, Vol. 59, 1203–1211, December 22, 1989 

Ex. D

# Direct Expression Cloning of Vascular Cell Adhesion Molecule 1, a Cytokine-Induced Endothelial Protein That Binds to Lymphocytes

Laurelee Osborn, Catherine Hession, Richard Tizard, Cornelia Vassallo, Stefan Luhowskyj, Gloria Chi-Rosso, and Roy Lobb
Biogen, Inc.
14 Cambridge Center
Cambridge, Massachusetts 02142

## Summary

**We have cloned a previously undescribed adhesion molecule, VCAM-1, which is induced by cytokines on human endothelial cells and binds lymphocytes. Using a novel method requiring neither monoclonal antibodies nor purified protein, VCAM-1–expressing clones were selected by adhesion to human lymphoid cell lines. VCAM-1 mRNA is present in endothelial cells at 2 hr after treatment with IL-1 or TNF-α and is maintained for at least 72 hr; leukocyte binding activity parallels mRNA induction. Cells transfected with VCAM-1 bind the human leukemia lines Jurkat, Ramos, Raji, HL60, and THP1, but not peripheral blood neutrophils. VCAM-1, which belongs to the immunoglobulin gene superfamily, may be central to recruitment of mononuclear leukocytes into inflammatory sites in vivo.**

## Introduction

Multiple lines of evidence indicate that inflammatory reactions are modulated by the interaction of circulating leukocytes with adhesion molecules on the luminal surface of blood vessels. These vascular cell adhesion molecules (VCAMs) arrest circulating leukocytes and thus perform the first step in their recruitment to infected or otherwise inflamed tissue sites. A growing body of evidence indicates that VCAMs may serve multiple functions, both normal and abnormal. They may play important roles in a wide range of pathological states involving cell–cell recognition, including tumor invasion and metastasis and viral infection, as well as various inflammatory syndromes such as tissue reperfusion damage and allograft rejection (for review see Harlan, 1985; Wallis and Harlan, 1986; Bevilacqua et al., 1987b; Cotran and Pober, 1988). Consequently, intensive effort has been devoted to their characterization.

To date, two cytokine-inducible VCAMs, intercellular adhesion molecule 1 (ICAM-1) and endothelial leukocyte adhesion molecule 1 (ELAM-1), have been cloned and characterized (Simmons et al., 1988; Staunton et al., 1988; Bevilacqua et al., 1989). ICAM-1 is found on many cell types, and its expression on vascular endothelium is strongly up-regulated by the inflammatory cytokines interleukin 1 (IL-1), tumor necrosis factor α (TNF-α), and interferon γ (IFN-γ), both in vitro and in vivo (Pober et al., 1986; Dustin and Springer, 1988; Cotran and Pober, 1988). Its ligand is lymphocyte function–associated antigen 1 (LFA-1), one of a trio of heterodimeric molecules known as the $\beta_2$ integrins or the CD11/18 family (Dustin et al., 1986; Rothlein et al., 1986; Marlin and Springer, 1987). ELAM-1, initially defined by a monoclonal antibody (MoAb) that partially blocks polymorphonuclear leukocyte (PMN) adhesion to cytokine-treated human umbilical vein endothelial cells (HUVECs), is a cell surface protein rapidly synthesized by HUVECs in response to the inflammatory cytokines IL-1 or TNF-α but not IFN-γ (Bevilacqua et al., 1987a).

Neither ELAM-1 nor ICAM-1 can account for the lymphocyte adhesion to cytokine-treated HUVECs observed in vitro. ELAM-1 is selective for PMNs, and perhaps monocytes, but does not bind lymphocytes (Bevilacqua et al., 1987a, 1989; our unpublished data). Although the ICAM-1–LFA-1 pathway does function in lymphocyte adhesion, MoAbs that block the LFA-1 pathway only weakly inhibit the binding of lymphocytic cells to activated endothelium (Haskard et al., 1986; Dustin and Springer, 1988), providing evidence for another pathway. Furthermore, in patients with a genetic deficiency of CD18 and hence of LFA-1, lymphocyte recruitment into inflammatory sites is normal, despite profound defects in recruitment of phagocytic cells (Anderson and Springer, 1987), indicative of an as yet uncharacterized lymphocyte migration pathway in vivo as well as in vitro.

We have functionally characterized the induction of a novel VCAM for lymphocytes. To clone the cDNA for this protein, we screened an IL-1–induced HUVEC expression library. To obviate the need for antibodies or purified protein with which to pan for transfected cells expressing the molecule, we developed a new screening procedure based on a functional assay for cell–cell adhesion, in conjunction with a subtraction technique to enrich our library for cDNAs of interest. We have used this method to clone both ELAM-1 and, as described here, a novel adhesion molecule for lymphocytes, VCAM-1.

## Results

### Lymphoid Cell Lines Bind to a Novel Adhesion Molecule on Cytokine-Treated HUVECs

We have recently cloned ELAM-1 (Hession et al., unpublished data), allowing us to examine the adhesion of human lymphoid cell lines to control and ELAM-1–transfected COS cells and to control and cytokine-treated HUVECs (Table 1). The T leukemia line Jurkat and EBV-transformed B cell line Ramos bind preferentially to induced HUVECs, as does the promyelocytic line HL60. The B lymphoblastoid line JY shows strong binding to untreated HUVECs, with slightly increased binding to cytokine-treated HUVECs, consistent with published data (Dustin and Springer, 1988). However, none of the lymphoid lines shows a preferential interaction with ELAM-1–transfected COS cells (Table 1), in agreement with the suggested role of ELAM-1 in PMN but not peripheral blood lymphocyte adhesion in vivo (Bevilacqua et al., 1987a, 1989).

To examine the contribution of the ICAM-1–LFA-1 path-

Table 1. Adhesion of Cell Lines to TNF-α–Induced HUVECs and ELAM-1–Transfected COS7 Cells

| | HUVEC (cells bound/mm²) | | COS7 (cells bound/mm²) | |
|---|---|---|---|---|
| Target Cell | Control | Induced | CDM8 | ELAM-1 |
| Jurkat | 40 ± 15 | 2816 ± 223 | 32 ± 4 | 54 ± 20 |
| Ramos | 16 ± 1 | 3240 ± 40 | 35 ± 5 | 60 ± 5 |
| JY | 2600 ± 125 | 3035 ± 66 | 215 ± 46 | 298 ± 4 |
| HL60 | 6 ± 1 | 2655 ± 155 | 65 ± 5 | 1635 ± 115 |

Target cells were incubated for 10 min at room temperature with HUVEC monolayers, either untreated (control) or treated with 10 ng/ml recombinant human TNF-α, or with COS7 monolayers that had been electroporated with CDM8 (vector) or ELAM-1/CDM8 72 hr previously. TNF-α treatment of HUVECs was for 24 hr except HL60, which was for 4 hr. Results are mean ± standard deviation of quadruplicate determinations in one experiment, representative of at least three experiments.

way to the adhesion of lymphoid cell lines to cytokine-treated HUVECs, the effect of a blocking MoAb was determined. MoAb 60.3, which binds to an epitope on the common β chain of the CD11/CD18 family to which LFA-1 belongs, completely inhibits the binding of JY cells to either control or cytokine-treated HUVECs (Figure 1A), consistent with published data showing that JY adhesion to HUVECs is entirely LFA-1 mediated (Dustin and Springer, 1988). In contrast, MoAb 60.3 has no effect on the adhesion of Ramos or Jurkat cells to cytokine-treated HUVECs (Figure 1A).

To characterize this interaction further, the cytokine sensitivity and time course of induction of Ramos and Jurkat cell adhesion were examined. Adhesion of either cell line occurs after treatment with IL-1 (10 U/ml) or TNF-α (10 ng/ml) but not IFN-γ (10 ng/ml) (data not shown). Adhesion of either cell line is detectable at 2 hr, reaches maximal levels at 4–6 hr, and is sustained for at least 48 hr (Jurkat; Figure 1B).

These data indicate that certain lymphoid cell lines bind to a novel cytokine-induced VCAM on endothelial cells. To clone this molecule, we used Ramos and Jurkat as representative cell lines for screening a direct expression library.

### Direct Expression Cloning of VCAM-1

cDNA was prepared from HUVECs treated for 2.5 hr with IL-1 and cloned into the expression vector CDM8 (Seed, 1987). This library was screened with a twice-subtracted cDNA probe of IL-1–induced HUVEC cDNA minus uninduced HUVEC mRNA, and a sublibrary was picked from this screen. The sublibrary was rescreened by hybridization with probes for ELAM-1 and ICAM-1, leaving 125 clones that hybridize with the subtracted cDNA but not with ELAM-1 or ICAM-1. These clones were pooled in groups of five and each pool was transfected into COS cells by spheroplast fusion; cells were then screened for adhesion to fluorescently labeled Ramos (or Jurkat) cells 48 hr following fusion. Ramos cells gave somewhat better results because the background binding to COS cells was consistently low, while Jurkat cells often gave high levels of background binding.

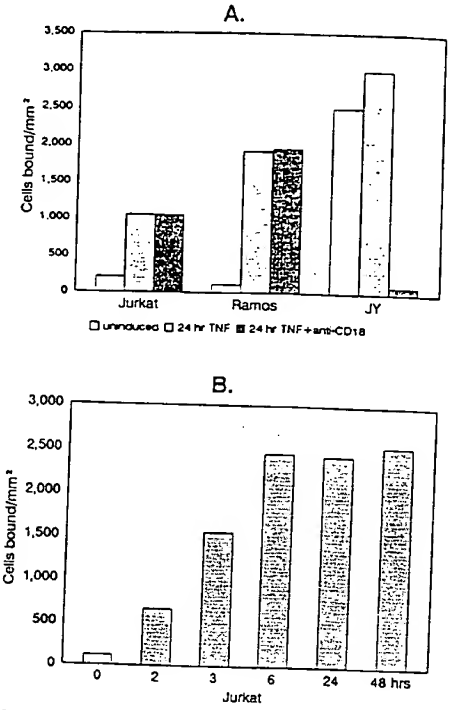


Figure 1. Binding of Ramos and Jurkat Cells to Cytokine-Induced HUVECs

(A) Anti-CD18 (MoAb 60.3) does not inhibit Ramos or Jurkat cell binding to TNF-α–treated HUVECs. Ramos, Jurkat, and JY cells were assayed for binding to control HUVECs and HUVECs treated with TNF-α for 24 hr as described in Experimental Procedures. Anti-CD18 treatment was performed by incubating target cells with MoAb 60.3 at a concentration of 10 μg/ml for 30 min at room temperature prior to assay.

(B) HUVEC binding to Jurkat cells is maintained at least 48 hr after TNF-α treatment. TNF-α at 10 ng/ml was added to HUVEC culture medium at the indicated time prior to assay. Results are the mean of quadruplicate measurements in one experiment, representative of at least three experiments; standard deviations were less than 15%.

Plates were scanned visually using a fluorescence microscope, and rosettes, consisting of Ramos cells adhering to a COS cell, were picked with a Rainin Pipetman (primary screen). Plasmid was rescued from each rosette, electroporated into bacteria (Dower et al., 1988), amplified, transfected into COS cells by spheroplast fusion, and rescreened with Ramos cells (secondary screen). One rosette of eight from the first primary screen yielded numerous large rosettes on the secondary screen (Figures 2a and 2b). Six individual plasmids from the original rosette were analyzed by gel electrophoresis, and the largest one, with a cDNA insert of 2.8 kb, was electroporated into COS cells; when panned with Ramos or Jurkat cells, large and

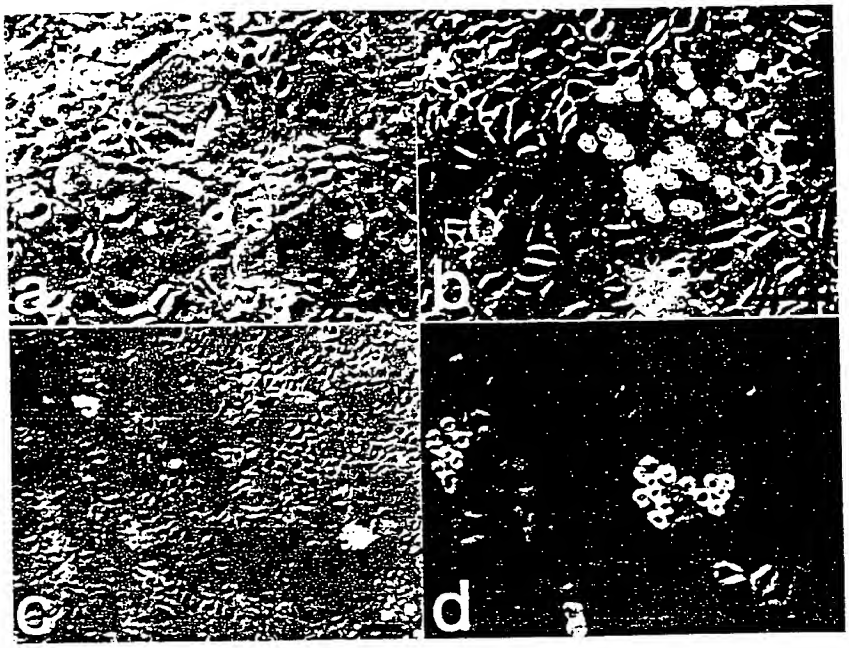


Figure 2. Functional Detection and Isolation of a VCAM-1 cDNA Clone

(a) COS cells transfected by spheroplast fusion with the induced HUVEC sublibrary were incubated with CFD-labeled Ramos cells and washed (primary screen); the indicated small rosette was picked with a Pipetman P200 (Rainin).

(b) Plasmid DNA rescued from the rosette in (a) was electroporated into bacterial host cells and transfected by spheroplast fusion into COS cells, which were incubated with CFD-labeled Ramos cells and washed as before (secondary screen); a representative of the resulting large rosettes is shown.

(c) Plasmid minipreps were prepared from six bacterial colonies obtained from electroporation of the DNA rescued from the rosette in (a). The plasmid with the largest insert size (2.8 kb) was electroporated into COS cells, which were panned as before. Numerous large rosettes were seen.

(d) VCAM-1/CDM8 electroporated into COS cells and panned with CFD-labeled Jurkat cells. Each bar represents 100 μm.

numerous rosettes were present (Figures 2c and 2d). This clone was designated VCAM-1/CDM8.

### VCAM-1 mRNA and Genomic DNA

The VCAM-1 cDNA was used as a probe to characterize the time course of induction and cell-type distribution of VCAM-1 mRNA. VCAM-1 mRNA is approximately 3200 nucleotides in length and is barely detectable in uninduced HUVECs or HUVECs treated for 1 hr with TNF-α, but levels increase greatly by 2 hr after induction by TNF-α and remain high for at least 72 hr (Figure 3A). These results are consistent with the time course of adhesion: binding is detectable 2 hr after cytokine treatment and is sustained for at least 48 hr (Figure 1B). This induction is somewhat slower and more long-lasting than that observed for ELAM-1 mRNA, which is maximally induced within 1 hr after cytokine treatment but drops significantly after 24 hr (Figure 3B).

RNA from several human cell lines and tissues was screened by Northern blotting for the presence of VCAM-1 sequences (data not shown). Untreated HL60 cells and HL60 cells treated with the differentiating agents retinoic acid and IFN-γ were included to determine if VCAM-1 mRNA could be induced in these cells; neither of the treatments induced VCAM-1 mRNA. Human erythroleukemia (HEL) cells, both treated with PMA and untreated, were included because these cells contain platelet α-granule–like organelles and express a number of proteins found in both platelets and endothelial cells (Tabilio et al., 1984); no VCAM-1 mRNA was observed in HEL cells. The highest concentrations of VCAM-1 mRNA were present in induced HUVECs, tonsil, and kidney; a very faint signal was detectable in placenta and no signal was present in liver. Hybridization of the VCAM-1 probe to human genomic DNA digested with various restriction enzymes gave a simple pattern consistent with the presence of one gene (data not shown).

### Nucleotide and Amino Acid Sequence

The cDNA insert of VCAM-1/CDM8 is 2811 bp in length,

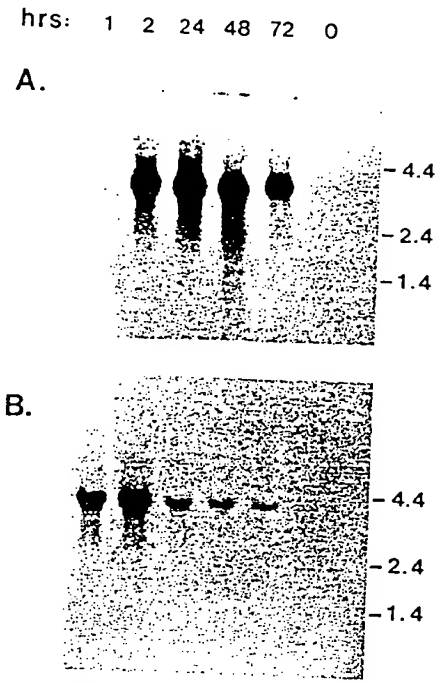


Figure 3. Kinetics of Induction of VCAM-1 and ELAM-1 mRNA in TNF-α–Treated Endothelial Cells

Recombinant human TNF-α was added to the medium of HUVECs, and after the indicated number of hours cells were harvested and total cellular RNA prepared. Ten micrograms of each sample was electrophoresed through an agarose–formaldehyde gel and transferred to Gene Screen (New England Nuclear/Du Pont). (A) The blot was hybridized with a radiolabeled VCAM-1 cDNA insert. (B) The same blot hybridized with a radiolabeled ELAM-1 cDNA insert. Molecular weight markers are indicated at right.

and contains 106 bp of 5′ untranslated sequence, a 1941 bp open reading frame, and 734 bp of 3′ untranslated sequence (Figure 4). Hydrophobicity analysis and comparison with known cleavage patterns predict a signal peptide of 24 amino acid residues (von Heijne, 1986; Figure 4). The mature protein is predicted to consist of a 582 amino acid extracellular domain, a 22 amino acid transmembrane region, and a short, 19 residue cytoplasmic tail, with a molecular weight of about 69,000. The extracellular domain contains six potential N-linked glycosylation sites; if these sites are glycosylated the predicted $M_r$ of VCAM-1 is about 90,000.

### Homologies to Other Proteins

The amino acid sequence of VCAM-1 was analyzed for homology to other proteins in the NBRF and NEW data bases using the program FASTP (Lipman and Pearson, 1985). All homologies detected were to proteins of the immunoglobulin (Ig) gene superfamily. Members of this superfamily are characterized by the presence of one or (usually) more Ig homology regions, each consisting of a disulfide-bridged loop that has a number of antiparallel β-pleated strands arranged in two sheets. Three types of Ig homology regions (C, V, and H, also known as C1, V, and C2) have been defined, each with a typical length and consensus of amino acid residues at certain positions relative to the two cysteine residues that form the bridge (for reviews see Hunkapillar and Hood, 1989; Williams and Barclay, 1988). By manual alignment, VCAM-1 was found to contain six Ig homology units of the H type (Figure 5). Units 2 and 4 are strikingly similar, as are units 3 and 5, indicating that VCAM-1 may have been increased in size at some point during its evolution by duplication of the 2–3 tandem unit. VCAM-1 belongs structurally to a subset of the superfamily that includes ICAM-1, neural cell adhesion molecule (NCAM), myelin–associated glycoprotein (MAG), biliary glycoprotein 1 (BGP-1), and carcinoembryonic antigen (CEA). The percentage of amino acid identity between VCAM-1 and these proteins in their most homologous regions as determined by FASTP and their ALIGN scores (Dayhoff et al., 1983) are shown in Table 2. Interestingly, FASTP did not detect ICAM-2 (Staunton et al., 1989) homology with VCAM-1, although ALIGN gives a score of 5 SD above random. Comparison of the VCAM-1 cDNA sequence with the GenBank data base revealed no significant homologies.

### Cell Adhesion to VCAM-1–Transfected COS Cells

Binding of a series of human leukocyte cell lines to COS cells transfected with control (CDM8) or VCAM-1/CDM8 plasmid DNA was examined (Table 3). As expected, Jurkat and Ramos cells bound selectively to VCAM-1–transfected cells, while JY cells did not. The promyelocytic cell line HL60 and the human monocytic cell line THP1 also bound to VCAM-1. Since HL60 cells bind well to ELAM-1 (Table 1), this suggests that these cells use at least two adhesion pathways to adhere to cytokine-treated HUVECs. PMNs showed no selective adhesion to VCAM-1–transfected COS cells (Table 3), despite binding well to ELAM-1–transfected cells in parallel assays. Both peripheral blood lymphocytes and T lymphoblasts show significant basal COS cell binding, making an examination of their specific adhesion to VCAM-1–transfected cells difficult. However, in preliminary experiments, MoAb 4B9, which reacts selectively with TNF-α–treated HUVECs and blocks Ramos cell adhesion (T. Carlos, B. Schwartz, and J. Harlan, personal communication), binds to VCAM-1 and partially inhibits the binding of both T lymphoblasts and peripheral blood lymphocytes to HUVECs treated with TNF-α for 24 hr (Lobb and Osborn, unpublished data). Taken jointly, these results suggest that VCAM-1 binds selectively to lymphocytes and perhaps monocytes but not to granulocytes.

## Discussion

Immunology, cancer biology, and the study of pattern formation during morphogenesis all have contributed to our

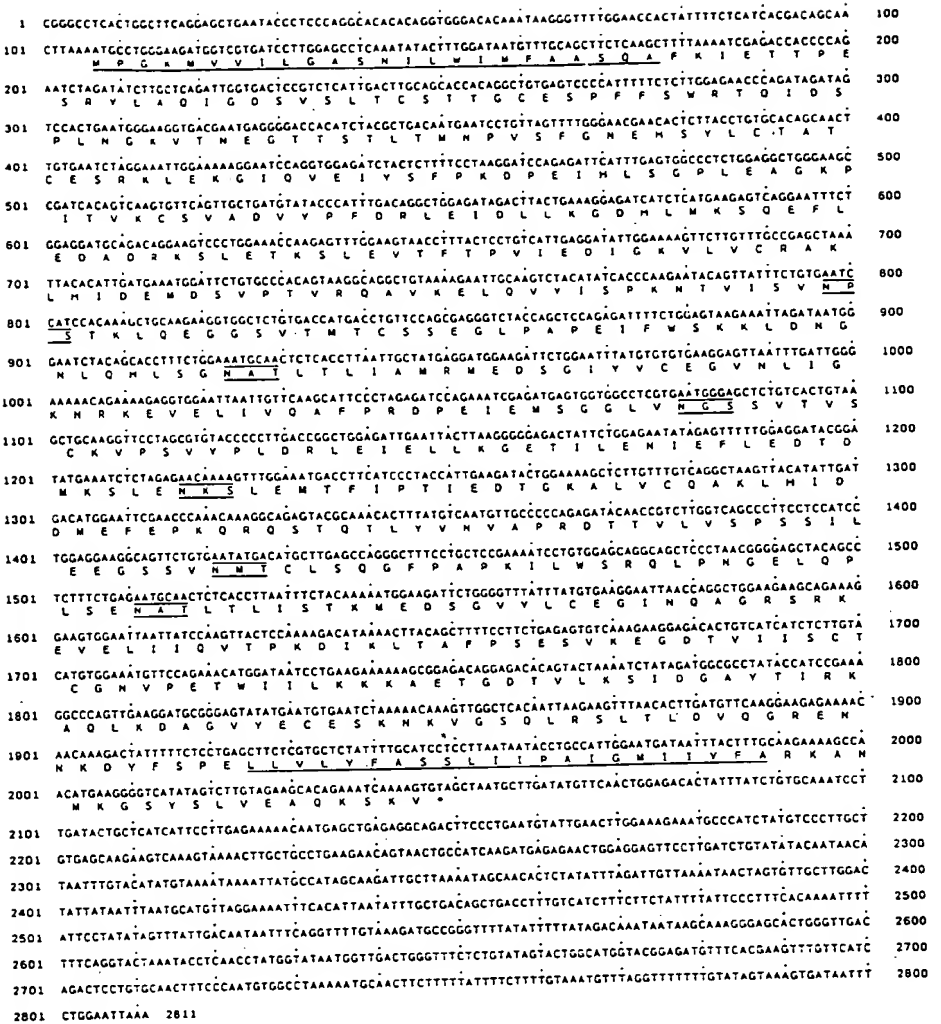

Figure 4. Nucleotide and Deduced Amino Acid Sequence of a VCAM-1 cDNA

The predicted signal sequence (beginning at nucleotide 107) and transmembrane region (beginning at nucleotide 1925) are underlined; potential N-linked glycosylation sites are both underlined and overlined.

understanding of the mechanisms by which expression of adhesion molecules influence both normal and pathological functions involving cell–cell recognition. Adhesion molecules often perform more than one normal function and have been implicated in pathological events such as viral infection and neoplastic cell migration and/or metastasis.

To date, the identification, characterization, and cloning of cellular adhesion molecules have required the generation of specific MoAbs or purification of quantities of protein or functional ligand. However, with the cloning of both ELAM-1 (Hession et al., unpublished data) and, as described here, VCAM-1, we have established a more general method for the direct expression cloning of adhesion molecules. Cell–cell adhesion screening detects clones that are, by definition, functional; more importantly, it requires no prior knowledge of the structure of the protein or its cognate ligand. We believe this method should be

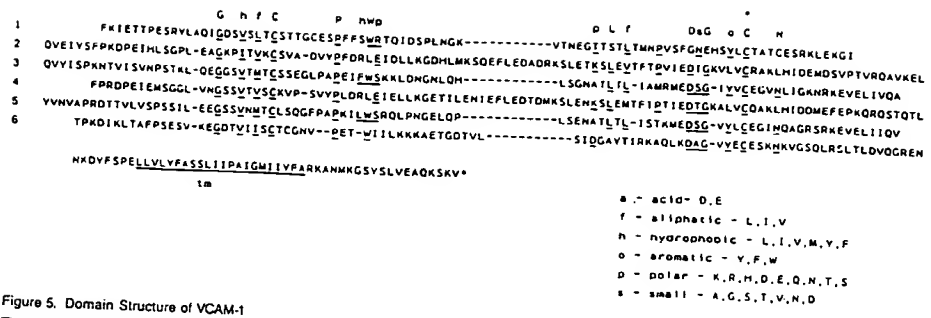


Figure 5. Domain Structure of VCAM-1

The sequence was manually aligned using conserved residues of Ig homology units of the H or C2 type (from Hunkapillar and Hood, 1989; residues conserved among many H-type Ig superfamily gene domains are indicated at the top of the figure and underlined in each domain). The transmembrane region (tm) is indicated. Note extensive homology of domain 2 with 4 and 3 with 5.

applicable to the cloning of other adhesion molecules, including other VCAMs.

Several lines of evidence are consistent with an important role for VCAM-1 in lymphocyte recruitment in vivo. First, VCAM-1 is rapidly induced by the key inflammatory cytokines IL-1 and TNF-α, and its induction is sustained for 48–72 hr. This time course parallels the sustained mononuclear leukocytic infiltration seen in classic delayed hypersensitivity reactions (Dvorak et al., 1980). Second, HUVECs in vitro have been shown to be a valid model for postcapillary venules in vivo, since both ICAM-1 and ELAM-1 are present both on cytokine-treated HUVECs and at inflammatory sites in vivo and are induced in vivo by local injection of cytokines (Cotran and Pober, 1988; Munro et al., 1989). Third, in frozen section assays of human synovium, lymphocytes bind selectively to inflamed vessels but not to normal vessels, consistent with the presence in vivo of lymphocyte-selective, induced VCAMs (Jalkanen et al., 1986). Taken jointly, these results suggest that VCAM-1 may be a central mediator of lymphocyte recruitment into inflammatory sites in vivo.

We are currently investigating the nature of the ligand of VCAM-1. We have found that VCAM-1–leukocyte binding is a temperature-sensitive, energy-requiring process (Lobb and Osborn, unpublished data), consistent with the interaction of VCAM-1 with an integrin, an attractive model by analogy with ICAM-1 binding to LFA-1, a $\beta_2$ integrin. $\beta_2$ integrins are not involved in binding of Jurkat or Ramos cells to VCAM-1, as assessed by the inability of MoAb 60.3 to inhibit their adhesion to induced HUVECs (Figure 1A) or VCAM-1–transfected cells (data not shown). Interestingly, it has been reported that high levels of CD29, the $\beta_1$ subunit shared by the very late antigen (VLA) group of integrins, are characteristic of a subset of lymphocytes enriched in chronic inflammatory sites such as the synovia of rheumatoid arthritis patients (Pitzalis et al., 1988). Preliminary experiments indicate that antibodies to CD29 can completely inhibit binding of Ramos to VCAM-1 (M. Elices, M. Hemler, R. Lobb, and L. Osborn, unpublished data), implicating one of the VLAs as a ligand for VCAM-1.

Multiple functions have been identified for the closest relatives of VCAM-1. ICAM-1, for example, is present on many cell types, including cytokine-induced endothelial cells, and normally functions as an accessory molecule aiding leukocyte adhesion and migration (Anderson and Springer, 1987; Dustin and Springer, 1988). In addition, ICAM-1, CEA, and nonspecific cross-reacting antigen are highly expressed on several types of tumor cells (Rogers, 1983; Johnson et al., 1989). This has led to speculation that ectopic expression of these adhesion molecules may

Table 2. Homology of VCAM-1 with Ig Superfamily Members

| Protein | Residues of Protein Homologous with VCAM-1 | Residues of VCAM-1 Homologous with Protein | Percent Identity | ALIGN Score |
|---|---|---|---|---|
| CEA | 483–615 | 211–337 | 29% | 7.7 |
| MAG | 101–410 | 275–595 | 26% | 6.9 |
| ICAM-1 | 290–357 | 95–165 | 26% | 10.1 |
| NCAM (chicken) | 5–360 | 40–399 | 19% | 3.0 |
| BGP-1 | 61–412 | 141–503 | 19% | 7.8 |

The VCAM-1 cDNA sequence was compared with sequences in the NEW data base and with ICAM-1 and ICAM-2 sequences entered manually (see text for ICAM-2 results). FASTP (Lipman and Pearson, 1985) was used to identify the most similar regions to VCAM-1 of each protein, and ALIGN (Dayhoff et al., 1983) was used to generate overall alignment scores. Sequences were taken from the following references: CEA, Oikawa et al. (1987); MAG, Lai et al. (1987); ICAM-1, Simmons et al. (1988) and Staunton et al. (1988); NCAM (chicken), Hemperly et al. (1986); BGP-1, Hinoda et al. (1988).

Table 3. Adhesion of Cell Lines to VCAM-1–Transfected COS7 Cells

| Target Cell | Cells Bound/mm$^2$ | |
|---|---|---|
| | CDM8 | VCAM-1 |
| Jurkat | 19 ± 4 | 780 ± 82 |
| Ramos | 7 ± 2 | 458 ± 63 |
| Raji | 2 ± 1 | 130 ± 24 |
| JY | 390 ± 125 | 476 ± 85 |
| HL60 | 9 ± 2 | 477 ± 68 |
| THP1 | 244 ± 11 | 1601 ± 56 |
| PMN | 10 ± 4 | 15 ± 4 |

Target cells were incubated for 10 min at room temperature with COS7 monolayers that had been electroporated with CDM8 (vector) or VCAM-1/CDM8 72 hr previously.

increase a tumor's ability to invade and to metastasize. Finally, several Ig superfamily members have been subverted to function as viral receptors: ICAM-1, CD4, and the poliovirus receptor allow infection of cells by rhinovirus, HIV, and poliovirus, respectively (reviewed in White and Littman, 1989). It will be of interest to determine if VCAM-1, like its nearest relatives, also has multiple functions in health and disease.

In sum, VCAM-1 is induced on the surface of endothelial cells within hours of stimulation by the inflammatory cytokines IL-1 or TNF-α and remains at high levels for at least 72 hr after induction. These characteristics are consistent with a role in recruiting immune-competent cells to sites of infection or aseptic inflammation; the induction kinetics indicate that VCAM-1 may play a role in both acute and chronic inflammation. The range of cells to which it binds suggests involvement in recruitment of both T and B lymphocytes and perhaps monocytes but not granulocytes. As discussed above, adhesion and recognition mechanisms gone awry may cause or contribute to a number of disease states involving differentiation or the immune system. Molecular characterization of adhesion pathways involved in normal tissue development and physiology will improve our ability to understand and perhaps to correct derangements of their function. In particular, VCAM-1–dependent pathways of leukocyte recruitment may provide new intervention points for the correction of pathologies associated with acute and chronic inflammation.

## Experimental Procedures

### Cells

Ramos, Jurkat, Raji, K562, HL60, JY, THP1, and COS7 cells were obtained from the American Type Culture Collection and maintained in RPMI, 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, except COS7 cells, which were grown in HEPES-buffered DMEM, 10% FBS, 2 mM glutamine. HEL cells were provided by Rodger P. McEvers and maintained in RPMI medium as above. HUVECs were isolated and subcultured as described (Gimbrone, 1976; Bevilacqua et al., 1985); they were used for adhesion assays or RNA isolation at passages 3–5. PMNs were isolated from anticoagulated blood by gradient centrifugation (Lymphocyte Separation Medium, Organon Teknika Co., Durham, NC), 3% dextran sedimentation, and hypotonic lysis (Boyum, 1968).

### cDNA Library Construction

RNA was isolated by the procedure of Chirgwin et al. (1979) from HUVECs treated with recombinant human IL-1β (10 U/ml, Biogen) for 2.5 hr, and poly(A)$^+$ RNA was isolated by two rounds of chromatography on oligo(dT)-cellulose (Pharmacia). A cDNA library was constructed in the expression vector CDM8 (Seed, 1987) by the method of Gubler and Hoffman (1983). A subtracted probe was made by hybridizing 0.2 μg of $^{32}$P-labeled induced HUVEC cDNA with 30 μg of uninduced HUVEC poly(A)$^+$ RNA, followed by HAP chromatography (Davis, 1986); this subtraction was then repeated. One million clones were plated on Gene Screen Plus filters (New England Nuclear), and replicate filters were screened with the subtracted probe as described previously (Cate et al., 1986). A sublibrary of 864 colonies (impure owing to the high density of plating; we estimate 5–10 clones per pick) was picked into microtiter wells. Sublibrary arrays were reprobed with the subtracted probe and with 30 base oligomers to ELAM-1 and ICAM-1 cDNA sequences; wells that were positive for hybridization with the subtracted probe but negative with ELAM-1 and ICAM-1 probes were inoculated in pools of five for spheroplast fusion (total of 25 pools).

### Cell–Cell Adhesion Screen

COS7 cells in 100 mm plates were transfected with the sublibrary pools by spheroplast fusion (Sandri-Goldin et al., 1981; Seed and Aruffo, 1987). Forty-eight hours later, they were screened with Ramos cells labeled with carboxyfluorescein diacetate (CFD) as follows. Cells were labeled by suspending at 5 × 10$^6$ cells per ml in RPMI, 1% FBS, and adding 5 μl per ml of cells of CFD (Calbiochem) dissolved in acetone at 20 mg/ml (Brenan and Parish, 1984). After incubation at 37°C for 15 min, cells were washed twice and suspended in RPMI, 1% FBS at 1 × 10$^6$ cells per ml. Labeled cells (6 ml) were added to each washed COS plate, incubated at room temperature for 15 min, and washed three or four times with RPMI, 1% FBS to remove nonadherent cells. The plates were then scanned by fluorescence microscopy for rosettes of adherent Ramos cells. The pools could not be unequivocally identified as positive or negative, so rosettes were marked on several plates by scratching the plastic dish with a needle, picked up under an inverted microscope using a Pipetman P200 (Rainin), and transferred to microfuge tubes. We estimate that about 300 cells were transferred per rosette. Since our spheroplast fusions typically yield 1%–3% transfectants in control experiments, each rosette picked should yield three to nine types of plasmid (each amplified in its host COS cell), if the spheroplast fusion results in transfer of one bacterial plasmid on average to each recipient COS cell. This estimate was consistent with analysis of individual colonies rescued from each rosette.

Hirt supernatants were prepared from each rosette essentially as in Seed and Aruffo (1987), by adding 400 μl of 0.6% SDS, 10 mM EDTA and gently vortexing; then 100 μl of 5 M NaCl was added, and the tubes were incubated overnight at 4°C. After spinning out debris, but before phenol–chloroform extraction, 10 μg of yeast tRNA was added as carrier. After precipitation and drying, the DNA was dissolved in 5 μl of water, and 2.5 μl of each DNA preparation was electroporated into the bacterial host for CDM8, MC1061/p3. Half of the bacteria were plated and half put into liquid culture overnight in LB, 12.5 μg/ml ampicillin, 7.5 μg/ml tetracycline. Approximately 1000 colonies were typically obtained from each rosette; the liquid culture was frozen in 15% glycerol and used later to inoculate cultures for secondary spheroplast fusion. A single positive clone was identified by two methods: a secondary screen using the liquid cultures as inoculum for spheroplast fusion and an analysis of six colonies from each of the eight rosettes picked. The secondary screen resulted in one clearly positive plate of the eight tested, with numerous, large rosettes. Restriction analysis of plasmid DNA from individual colonies followed by electroporation into COS cells of miniprep DNA from those colonies containing the highest molecular weight cDNA insert also resulted in a single positive clone, VCAM-1/CDM8, from the same rosette that was positive in the secondary screen.

### DNA Sequencing

The cDNA insert of VCAM-1/CDM8 was subcloned into vector PNN11 and sequenced by the method of Maxam and Gilbert (1980); the sequence was determined for 100% of both strands.

### RNA and DNA Blot Hybridization

Total or poly(A)$^+$ RNA was loaded onto 1% agarose–formaldehyde gels, electrophoresed, transferred onto Gene Screen filters, UV cross-linked as described (Church and Gilbert, 1984; Cate et al., 1986), and hybridized with the indicated probes, labeled by random oligomer priming (Feinberg and Vogelstein, 1983). The washed filters were subjected to autoradiography.

### Adhesion Assay

HUVECs were plated in 48-well clusters, grown to confluence, and treated with recombinant human IL-1β at 10 U/ml or TNF-α (Biogen) at 10 ng/ml for 24 hr prior to the adhesion assay. COS cells were transfected by electroporation: 10 μg of VCAM-1/CDM8 or CDM8 control plasmid DNA and 200 μg of sonicated salmon sperm DNA was dissolved in 800 μl of HeBS (20 mM HEPES [pH 7.0], 137 mM NaCl, 5 mM KCl, 0.7 mM $Na_2HPO_4$, 6 mM dextrose) and added to a pellet of $10^7$ COS cells that had been grown to 80% confluence and harvested by trypsinization. The cell suspension was tranferred to an electroporation cuvette (Bio–Rad) and electroporated at 280 V, 960 μF with a Bio–Rad Gene Pulser. After 10 min, cells were washed in complete medium, resuspended in complete medium with gentamycin, and plated at $10^5$ cells per cm$^2$ into 48 well plates. They were confluent after 48 hr and were used after 48 or 72 hr. In control experiments (electroporation of CD4/CDM8 followed by FACS analysis for surface CD4) the transfection efficiency was 5%–25% of surviving cells using this method. Suspension cells (Ramos, Jurkat, Raji, HL60, JY, THP1) were labeled for 18 hr with 1–2 μCi/ml [$^{35}$S]methionine or [$^3$H]thymidine added to their normal medium, washed twice prior to assay, and resuspended at 2–3 × $10^6$ per ml. Adhesion assays were performed as described (Bevilacqua et al., 1987a); adherent radiolabeled cells were quantified by liquid scintillation counting, PMNs by endogenous myeloperoxidase content using standard assays.

### Acknowledgments

We thank John Harlan for MoAbs 60.3 and 4B9 and for access to unpublished information, Brian Seed for vector CDM8, Ping Chow for computer analysis of protein sequence homologies, Susan Goelz for helpful discussions, and Mark Wysk, Janice DeMarinis, and Bridget Eberhardt for excellent technical assistance.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 6, 1989; revised October 27, 1989.

### References

Anderson, D. C., and Springer, T. A. (1987). Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1, and p150,95 glycoproteins. Annu. Rev. Med. *38*, 175–194.

Bevilacqua, M. P., Pober, J. S., Wheeler, M. E., Cotran, R. S., and Gimbrone, M. A., Jr. (1985). Interleukin 1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leukocytes, monocytes, and related leukocyte cell lines. J. Clin. Invest. *76*, 2003–2011.

Bevilacqua, M. P., Pober, J. S., Mendrick, D. L., Cotran, R. S., and Gimbrone, M. A., Jr. (1987a). Identification of an inducible endothelial–leukocyte adhesion molecule. Proc. Natl. Acad. Sci. USA *84*, 9238–9242.

Bevilacqua, M. P., Wheeler, M. E., Pober, J. S., Fiers, W., Mendrick, D. L., Cotran, R. S., and Gimbrone, M. A., Jr. (1987b). Endothelial-dependent mechanisms of leukocyte adhesion: regulation by interleukin-1 and tumor necrosis factor. In Leukocyte Emigration and Its Sequelae, H. Movat, ed. (Basel: Karger), pp. 79–93.

Bevilacqua, M. P., Stengelin, S., Gimbrone, M. A., Jr., and Seed, B. (1989). Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. Science *243*, 1160–1165.

Boyum, A. (1968). Isolation of mononuclear cells and granulocytes from blood. Scand. J. Clin. Lab. Invest. (Suppl. 97) *21*, 77–89.

Brenan, M., and Parish, C. R. (1984). Intracellular fluorescent labelling of cells for analysis of lymphocyte migration. J. Immunol. Meth. *74*, 31–38.

Cate, R. L., Mattaliano, R. J., Hession, C., Tizard, R., Farber, N. M., Cheung, A., Ninfa, E. G., Frey, A. Z., Gash, D. J., Chow, E. P., Fisher, R. A., Bertonis, J. M., Torres, G., Wallner, B. P., Ramachandran, K. L., Ragin, R. C., Manganaro, T. F., MacLaughlin, D. T., and Donahoe, P. K. (1986). Isolation of the bovine and human genes for Müllerian inhibiting substance and expression of the human gene in animal cells. Cell *45*, 685–698.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry *18*, 5294–5299.

Church, G. M., and Gilbert, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA *81*, 1991–1995.

Cotran, R. S., and Pober, J. S. (1988). Endothelial activation: its role in inflammatory and immune reactions. In Endothelial Cell Biology, N. Simionescu and M. Simionescu, eds. (New York: Plenum Press), pp. 335–347.

Davis, M. M. (1986). Subtractive cDNA hybridization and the T-cell receptor genes. In Handbook of Experimental Immunology, Vol. 2, 4th ed., A. M. Weir, ed. (Oxford: Blackwell Scientific Publications, Inc.), pp. 1–13.

Dayhoff, M. O., Barker, W. C., and Hunt, L. T. (1983). Establishing homologies in protein sequences. Meth. Enzymol. *91*, 524–545.

Dower, W. J., Miller, J. F., and Ragsdale, C. W. (1988). High efficiency transformation of *E. coli* by high voltage electroporation. Nucl. Acids Res. *16*, 6127–6145.

Dustin, M. L., and Springer, T. A. (1988). Lymphocyte function–associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. J. Cell Biol. *107*, 321–331.

Dustin, M. L., Rothlein, R., Bhan, A. K., Dinarello, C. A., and Springer, T. A. (1986). Induction by IL1 and interferon-gamma: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). J. Immunol. *137*, 245–254.

Dvorak, H. F., Galli, S. J., and Dvorak, A. M. (1980). Expression of cell-mediated hypersensitivity in vivo—recent advances. Int. Rev. Exp. Pathol. *21*, 199–195.

Feinberg, A. P., and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. *132*, 6–13.

Gimbrone, M. A., Jr. (1976). Culture of vascular endothelium. Prog. Hemost. Thromb. *3*, 1–28.

Gubler, U., and Hoffman, B. J. (1983). A simple and very efficient method for generating cDNA libraries. Gene *25*, 263–269.

Harlan, J. M. (1985). Leukocyte–endothelial interactions. Blood *65*, 513–525.

Haskard, D., Cavender, D., Beatty, P., Springer, T., and Ziff, M. (1986). T lymphocyte adhesion to endothelial cells: mechanisms demonstrated by anti-LFA-1 monoclonal antibodies. J. Immunol. *137*, 2901–2906.

Hemperly, J. J., Murray, B. A., Edelman, G. M., and Cunningham, B. A. (1986). Sequence of a cDNA clone encoding the polysialic acid-rich and cytoplasmic domains of the neural cell adhesion molecule N-CAM. Proc. Natl. Acad. Sci. USA *83*, 3037–3041.

Hinoda, Y., Neumaier, M., Hefta, S. A., Drzeniek, Z., Wagener, C., Shively, L., Hefta, L. J. F., Shively, J. E., and Paxton, R. J. (1988). Molecular cloning of a cDNA coding biliary glycoprotein I: primary structure of a glycoprotein immunologically crossreactive with carcinoembryonic antigen. Proc. Natl. Acad. Sci. USA *85*, 6959–6963.

Hunkapillar, T., and Hood, L. (1989). Diversity of the immunoglobulin gene superfamily. Adv. Immunol. *44*, 1–63.

Jalkanen, S., Steere, A. C., Fox, R. I., and Butcher, E. C. (1986). A distinct endothelial cell recognition system that controls lymphocyte traffic into inflamed synovium. Science *233*, 556–558.

Johnson, J. P., Stade, B. G., Holzmann, B., Schwable, W., and Riethmuller, G. (1989). De novo expression of intercellular-adhesion molecule 1 in melanoma correlates with increased risk of metastasis. Proc. Natl. Acad. Sci. USA *86*, 641–644.

Lai, C., Brow, M. A., Nave, K. A., Noronha, A. B., Quarles, R. H., Bloom, F. E., Milner, R. J., and Sutcliffe, J. G. (1987). Two forms of 1B236/myelin-associatec glycoprotein, a cell adhesion molecule for postnatal neural development, are produced by alternative splicing. Proc. Natl. Acad. Sci. USA *84*, 4337–4341.

Lipman, D. J., and Pearson, W. R. (1985). Rapid and sensitive protein similarity searches. Science *227*, 1435–1441.

Martin, S. D., and Springer, T. A. (1987). Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function–associated antigen 1 (LFA-1). Cell *51*, 813–819.

Maxam, A. M., and Gilbert, W. (1980). Sequencing end-labeled DNA with base-specific chemical cleavages. Meth. Enzymol. *65*, 499–560.

Munro, J. M., Pober, J. S., and Cotran, R. S. (1989). Tumor necrosis factor and interferon-γ induce distinct patterns of endothelial activation and leukocyte accumulation in skin of *Papio anubis*. Am. J. Pathol. *135*, 121–133.

Oikawa, S., Nakazato, H., and Kosaki, G. (1987). Primary structure of human carcinoembryonic antigen (CEA) deduced from cDNA sequence. Biochem. Biophys. Res. Commun. *142*, 511–518.

Pitzalis, C., Kingsley, G., Haskard, D., and Panayi, G. (1988). The preferential accumulation of helper–inducer T lymphocytes in inflammatory lesions: evidence for regulation by selective endothelial and homotypic adhesion. Eur. J. Immunol. *18*, 1397–1404.

Pober, J. S., Gimbone, M. A., Jr., Lapierre, L. A., Mendrick, D. L., Fiers, W., Rothlein, R., and Springer, T. A. (1986). Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor, and immune interferon. J. Immunol. *137*, 1893–1896.

Rogers, G. T. (1983). Carcinoembryonic antigens and related glycoproteins: molecular aspects and specificity. Biochim. Biophys. Acta *695*, 227–249.

Rothlein, R., Dustin, M. L., Marlin, S. D., and Springer, T. A. (1986). A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. J. Immunol. *137*, 1270–1274.

Sandri-Goldin, R. M., Goldin, A. L., Levine, M., and Glorioso, J. C. (1981). High-frequency transfer of cloned herpes simplex virus type 1 sequences to mammalian cells by protoplast fusion. Mol. Cell. Biol. *8*, 743–752.

Seed, B. (1987). An LFA-3 cDNA encodes a phospholipid linked membrane protein homologous to its receptor CD2. Nature *329*, 840–842.

Seed, B., and Aruffo, A. (1987). Molecular cloning of the human CD2 antigen by a rapid immunoselection procedure. Proc. Natl. Acad. Sci. USA *84*, 3365–3369.

Simmons, D., Makgoba, M. W., and Seed, B. (1988). ICAM, an adhesion ligand of LFA-1, is homologous to the neural cell adhesion molecule NCAM. Nature *331*, 624–627.

Staunton, D. E., Martin, S. D., Stratowa, C., Dustin, M. L., and Springer, T. A. (1988). Primary structure of ICAM-1 demonstrates interaction between members of the immunoglobulin and integrin supergene families. Cell *52*, 925–933.

Staunton, D. E., Dustin, M. L., and Springer, T. A. (1989). Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. Nature *339*, 61–64.

Tabilio, A., Rosa, J.-P., Testa, U., Kieffer, N., Nurden, A. T., Del Canizo, M. C., Breton-Gorius, J., and Vainchenker, W. (1984). Expression of platelet membrane glycoproteins and α-granule proteins by a human erythroleukemia cell line (HEL). EMBO J. *3*, 453–459.

von Heijne, G. (1986). A new method for predicting signal sequence cleavage sites. Nucl. Acids Res. *14*, 4683–4690.

Wallis, W. J., and Harlan, J. M. (1986). Effector functions of endothelium in inflammatory and immunologic reactions. Pathol. Immunopathol. Res. *5*, 73–103.

White, J. M., and Littman, D. R. (1989). Viral receptors of the immunoglobulin superfamily. Cell *56*, 725–728.

Williams, A. F., and Barclay, A. N. (1988). The immunoglobulin superfamily—domains for cell surface recognition. Annu. Rev. Immunol. *6*, 381–405.

### GenBank Accession Number

The accession number for the sequence reported in this paper is M30257.